PB83-115170

Type A Viral Hepatitis
Effect of Chlorine on Infectivity

Rush-Presbyterian-St. Luke's Medical Center
Chicago, IL

Prepared for

Municipal Environmental Research Lab.
Cincinnati, OH

Oct 82

U.S. Department of Commerce
National Technical Information Service
NTIS

PB83-115170

EPA-600/2-82-089
October 1982

TYPE A VIRAL HEPATITIS: EFFECT OF CHLORINE ON INFECTIVITY

by

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Grant Number R805986-02-2

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CINCINNATI, OHIO 45268

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TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. RECIPIENT'S ACCESSION NO.
EPA-600/2-82-089		PB83 115170

4. TITLE AND SUBTITLE	5. REPORT DATE
Type A Viral Hepatitis: Effect of Chlorine on Infectivity	October 1982
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
David A. Peterson, Ph.D.	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
Rush-Presbyterian-St. Lukes Medical Center Chicago, Illinois 60612	BNC1A
	11. CONTRACT/GRANT NO.
	R-805986
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Municipal Environmental Research Laboratory, USEPA Drinking Water Research Division 26 West St. Clair St. Cincinnati, Ohio 45268	Final - 8/1/78-12/20/81
	14. SPONSORING AGENCY CODE
	EPA/600/14

15. SUPPLEMENTARY NOTES
Project Officer - John C. Hoff, Ph.D. (Phone: 513-684-7331)

16. ABSTRACT

The objective of this study was to determine the effect of (HOCl) treatment on the infectivity of hepatitis A virus (HAV). Prodromal chimpanzee feces, shown to induce hepatitis in marmosets (Saginus sp.), was clarified (JA 20/8K/30 min/5° C), the virus precipitated with 7% PEG 6000, harvested (JA 20/8K/30 min/5°C) and resuspended. The suspensions were layered onto 5-30% linear surcrose gradients, centrifuged (SW27/25K/3 hrs/5° C) and the fractions containing HAV were pooled, dialyzed and ampulized. A 1:500,000 dilution of this preparation induced hepatitis in 2/4 and seroconversion in 2/4 marmosets. A 1:50 dilution of this preparation that routinely produced hepatitis in 100% of the marmosets inoculated served as inoculum for subsequent experiments.

Disinfection experiments were conducted in 5°C and pH 7.0. The summary of results from different chlorine treatment experiments were as follows. HAV exposed to 0.5 mg/L free residual chlorine for 15 and 30 min produced hepatitis in 2/14 marmosets and seroconversion in 4/14. Treatment of HAV with 1 mg/L free residual chlorine for 30 and 60 min resulted in hepatitis in 1/12 animals and 4/12 animals seroconverted. HAV treated with 1.5 mg/L free residual chlorine for 30 min induced hepatitis and seroconversion in 1/10 marmosets. Thus, 0.5, 1.0 and 1.5 mg/L free residual chlorine decreased the incidence of overt hepatitis and seroconversion rates. Treatment with 2.0 and 2.5 mg/L free residual chlorine destroyed the infectivity of this partially purified HAV preparation.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field Group

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
Release to Public	Unclassified	29
	20. SECURITY CLASS *(This page)*	22. PRICE
	Unclassified	

EPA Form 2220-1 (9-73)

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FOREWARD

The U.S. Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimonies to the deterioration of our natural environment. The complexity of that environment and the interplay of its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution; it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems to prevent, treat, and manage wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, to preserve and treat public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research and provides a most vital communications link between the researcher and the user community.

In this study, the effect of chlorine on the infectivity of hepatitis A virus for marmosets was investigated. The report provides previously unavailable data on the resistance of this important waterborne pathogen to inactivation by disinfection. This information will be useful to those responsible for assuring the adequate treatment of public drinking water supplies.

Francis T. Mayo, Director
Municipal Environmental Research
Laboratory

ABSTRACT

The objective of this study was to determine the effect of chlorine (HOCl) treatment on the infectivity of hepatitis A virus (HAV). Prodromal chimpanzee feces, shown to induce hepatitis in marmosets (Saguinus sp.), was clarified (JA 20/8K/30 min/5°C), the virus precipitated with 7% PEG 6000, harvested (JA 20/8K/30 min/5°C) and resuspended. The suspensions were layered onto 5-30% linear sucrose gradients, centrifuged (SW27/25K/3 hrs/5°C) and the fractions containing HAV were pooled, dialyzed and ampulized. A 1:500,000 dilution of this preparation induced hepatitis in 2/4 and seroconversion in 2/4 marmosets. A 1:50 dilution of this preparation that routinely produced hepatitis in 100% of the marmosets inoculated served as inoculum for subsequent experiments.

Disinfection experiments were conducted at 5°C and pH 7.0. The summary of results from different chlorine treatment experiments were as follows. HAV exposed to 0.5 mg/L free residual chlorine for 15 and 30 min produced hepatitis in 2/14 marmosets and seroconversion in 4/14. Treatment of HAV with 1 mg/L free residual chlorine for 30 and 60 min resulted in hepatitis in 1/12 animals and 4/12 animals seroconverted. HAV treated with 1.5 mg/L free residual chlorine for 30 min induced hepatitis and seroconversion in 1/10 marmosets. Thus, 0.5, 1.0 and 1.5 mg/L free residual chlorine decreased the incidence of overt hepatitis and seroconversion rates. Treatment with 2.0 and 2.5 mg/L free residual chlorine destroyed the infectivity of this partially purified HAV preparation.

This report was submitted in fulfillment of Contract No. R805986-02-2 by Rush-Presbyterian-St. Luke's Medical Center under the sponsorship of the U.S. Environmental Protection Agency. This report covers a period from August 1, 1978 to October 31, 1981 and work was completed as of October 30, 1981.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

anti-HAV	-- antibodies to hepatitis A virus
CDF	-- chlorine demand free
dpi	-- days post inoculation
HA	-- hepatitis A
HAV	-- hepatitis A virus
HAV Ag	-- hepatitis A antigen(s)
HB	-- hepatitis B
HBV	-- hepatitis B virus
IM	-- intramuscular inovulation
MID_{50}	-- marmoset infectious dose 50% end point
NAnB	-- non A-non B hepatitis
PBS	-- phosphate buffered saline
RIA	-- Radioimmunoassay

SYMBOLS

Cs Cl	-- cesium chloride
DPD	-- N,N-diethyl-p-phenylenediamine
HOCl	-- hypochlorous acid
^{125}I	-- iodine 125 iosotope
PEG	-- polyethylene glycol

ACKNOWLEDGEMENTS

I wish to thank the Board of Health, City of Chicago, for providing space for housing most of our experimental animals.

SECTION 1

INTRODUCTION

In the United States viral hepatitis ranks fourth among reportable infectious diseases and is the most common, serious infectious disease for which no specific treatment exists. Viral hepatitis is caused by two distinctly different viruses, hepatitis A virus (HAV) and hepatitis B virus (HBV). From 1972 to 1976 about 40,000 to 50,000 cases of hepatitis A (HA) and 6,000 to 9,000 cases of hepatitis B (HB) have been reported annually in the United States (1), but the actual incidence of clinical disease and subclinical infection is undoubtedly several times greater than the number of reported cases (2). In addition, 80% to 90% of patients with posttransfusion hepatitis do not develop antibodies to either HAV or HBV, suggesting the existence of a third type of non A-non B viral hepatitis (NANB) caused by two as yet unidentified agents (3-5).

The fecal-oral transmission of HAV by contamination of water supplies, food and drink is well documented by the many detailed reports (6-10) of hepatitis A (HA) epidemics. HBV transmission generally occurs by parenteral inoculation of blood or blood products or by close family association (6) while NANB hepatitis is currently recognized primarily in the posttransfusion situation and its "natural" mode of transmission is probably very similar to that of HBV.

Waterborne outbreaks of HA could be prevented by treatment of potable water with an effective virucidal agent. Chlorine (hypochlorous acid [HOCl]), generally accepted as a universal water disinfectant, has been shown to be effective against adenovirus and various enteroviruses (11-21). Although Neefe et al. (17) showed that large amounts of chlorine destroyed infectivity of HAV in crude fecal filtrates, the effect of chlorine has not been extensively examined, primarily because an *in vitro* system for propagating HAV has only recently been identified (22, 23). As an alternative to studies *in vitro*, the effect of chlorine on HAV infectivity can be studied in a susceptible animal model. The virologic, serologic and pathologic findings that characterize HAV infections and type A hepatitis in human patients can be reproduced experimentally in marmoset monkeys.

The objective of this study was to determine the effect of chlorine (HOCl) treatment on the infectivity of hepatitis A virus (HAV) derived from fecal material.

SECTION 2

CONCLUSIONS AND RECOMMENDATION

Treatment of HAV with 0.5, 1.0 and 1.5 mg/L free residual chlorine decreased the development of overt hepatitis and seroconversion in marmosets when compared with controls receiving untreated virus. HAV treated with 2.0 and 2.5 mg/L free residual chlorine failed to induce hepatitis or seroconversion in inoculated marmosets. These results suggest that chlorine treatment levels of greater than or equal to 1.5 mg/L free residual chlorine for 30 min (5°C, pH 7.0) will effectively decrease the infectivity of HAV by more than four orders of magnitude. Data from a number of studies indicate that under these conditions other enteroviruses are reduced by two orders of magnitude in less than 5 min. The results of this investigation suggest that HAV may be considerably more resistant to chlorine inactivation than other enteroviruses.

With the recent development of *in vitro* cell culture systems for the propagation of HAV (22, 23) more extensive and precise research on this virus is now feasible and should be pursued. What are the optimal pH and temperature for treatment? What are the optimal exposure times and treatment levels for various chlorine species and for other disinfectants used in drinking water treatment? What interrelationships do these parameters of pH, temperature, exposure time and treatment levels have? What are the effects of viral aggregates or the presence of virus in water with a large chlorine demand? These are a few of the many unanswered questions concerning the parameters involved in inactivation of HAV by chlorine and other disinfectants. Further studies utilizing HAV in cell culture systems can answer many of these questions and refine further the chlorine treatment conditions necessary to keep potable water free of infectious HAV.

SECTION 3

MATERIALS AND METHODS

A. Biological Resources

i. Animals: Adult wild-caught and/or colony-born and reared white-lipped marmosets (Saguinus fuscicollis, S. nigricollis) and/or red-bellied marmosets (S. labiatus labiatus) were divided into experimental groups of 4 to 6 animals for baseline evaluation of serum transaminase levels and liver histology. Marmosets were inoculated by the intramuscular (IM) route. Development of infection and induction of hepatitis were monitored by weekly serum transaminase levels, liver biopsies performed at two-week intervals, and development of antibodies to HAV (anti-HAV) as previously described (24-28). The experimental period was 120 days for animals inoculated with untreated preparations and 150 days for those inoculated with HOCl treated preparations. Marmosets were maintained by procedures established in our colony (29-36).

ii. HAV source material: In previous work a chimpanzee (Ch. 122) was inoculated with human acute-phase hepatitis feces (GBG-BM isolate) and developed acute hepatitis 21 days post inoculation (dpi). Stool specimens were obtained daily and frozen at -70°C. Examination of these stool specimens for HAV antigen (HAV Ag) by radioimmunoassay and by electron microscopic evaluation revealed high levels of HAV in feces obtained from Ch 122 from 11-18 dpi. A 20% fecal pool prepared from stools collected on 13, 14, and 15 dpi induced hepatitis in 5 of 6 and seroconverted 6 of 6 marmosets inoculated. This pool served as the HAV source for this study.

iii. Serum:

b. "Normal" human and/or marmoset sera, lacking anti-HAV, was used as diluent and/or control sera as needed.

c. Anti-HAV sera, demonstrating a high titer of anti-HAV (greater than or equal to 1:20,000 for human sera; greater than or equal to 1:5,000 for marmoset sera), obtained from convalescent humans and marmosets was used to assay for HAV Ag. Partially purified gamma globulin was prepared by ammonium sulfate precipitation and DEAE-cellulose chromatography (DE 52, Whatman Ltd. England) and labeled with ^{125}I for RIA (37).

B. Partial purification of HAV.

Hepatitis A virus was partially purified from chimpanzee (prodromal phase) feces by the method of Moritsugu et al. (38).

i. Preparation of fecal extract: To 235 grams of feces in a 500 ml polycarbonate screw cap bottle with 30 g of glass beads (4 mm diameter, coated with PBS - 2% fetal calf serum, pH 7.4) and 250 ml of 0.01 M tris buffer, pH 8.0 and shaken rigorously for several min. HAV was recovered from the supernatant after clarification (2,700 rpm/45 min/4°C); the amount of supernatant withdrawn was replaced by fresh buffer and the extraction repeated. The pooled supernatant obtained after 4 extractions (approximately 500 ml) was clarified (10,000 rpm/60 min/5°C/JA 21 rotor), and the resulting supernatant (designated C-1 and stored at -70°C) served as starting material for further purification.

ii. Ultracentrifuge separations:

a. Rate-zonal separation on linear preformed sucrose gradients. C-1 material was concentrated approximately 100 fold by two pelleting steps. The first pellet (20,000 rpm/16 hours/5°C/JA 21 rotor) was resuspended in 1/10 the original volume of 0.01 M tris buffer, clarified (15,000 rpm/45 min/5°C/30 rotor) and the supernatant fluid labeled C-2. The second pelleting (29,000 rpm/14 hours/5°C/30 rotor) was resuspended in 1/10 of the original volume of C-2 in 0.01 M tris buffer, clarified (15,000 rpm/45 min/5°C/30 rotor) and labeled C-3. This material (C-3) was separated in a linear sucrose gradient (5% to 30% in 0.01 M tris buffer, pH 8.0 with 0.1% Nonidet P-40 [NP-40]). After centrifugation (26,000 rpm/180 min/5°C/SW 27 rotor) the gradients were fractionated and fractions examined by radioimmunoassay (RIA) for HAV Ag. Fractions in which HAV was found were pooled, the virus sedimented (38,000 rpm/120 min/5°C/SW 41 rotor) and resuspended in 0.05 M phosphate buffer (described below).

b. Isopycnic banding in cesium chloride (CsCl) followed by rate-zonal purification in linear sucrose gradients. Two and one-half milliliters of the C-2 supernatant (ii.a. above) were placed on top of a linear CsCl density gradient (1.10 to 1.5 g/cm^3 in 0.01 M tris buffer, pH 8.0 with 0.1% of NP-40) prepared in a SW 41 rotor centrifuge tube. After centrifugation (35,000 rpm/18 hours/5°C/SW 41 rotor) the gradients were fractionated, fractions examined by RIA and the 1.29-1.33 g/cm^3 and 1.39-1.41 g/cm^3 peaks pooled and dialyzed (24 hours/5°C/0.01 M tris pH

8.0/4 changes). This material was further purified on a linear sucrose gradient as described in B. ii. a. above.

iii. Freon extraction: C-1 material was extracted twice with Freon 113, the aqueous phase harvested after centrifugation (3500 rpm/5°C/30 min/JA 20 rotor), filtered (prefilter/800 nm/450 nm), filtrate concentrated by hydroextraction and placed on 5% to 30% linear sucrose gradient as described in B. ii. a. above.

iv. PEG extraction: C-1 was clarified by centrifugation (8,000 rpm/5°C/30 min/JA 20 rotor), the supernatant made up to 7% PEG 6000, the solution mixed periodically and incubated at 4°C overnight. The precipitate was harvested by centrifugation (8,000 rpm/5°C/30 min/JA 20 rotor) and resuspended in tris buffer and placed on 5% to 30% linear sucrose gradients as described in B. ii. a. above.

C. Chlorine inactivation experiments.

i. Glassware. The glassware was thoroughly cleaned and rinsed with glass-distilled, deionized water.

ii. Chlorine water. Stock solutions of sodium hypochlorite were prepared from Clorox (Clorox Corporation, Oakland, Ca.). Chlorinated water was prepared by adding 5 mg/L of a hypochlorite solution (prepared from Clorox) to glass-distilled, deionized water. The chlorinated water was allowed to stand at room temperature for one week before use.

iii. Buffers. All buffers were prepared with chlorinated water (described above) to produce chlorine demand-free buffers (CDF buffers). The buffer system for the inactivation experiments consisted of a 0.05 M phosphate buffer (KH_2PO_4-K_2HPO_4) prepared at pH 7. Buffers were exposed to ultraviolet light for 48 to 72 hours and then tested for chlorine by the orthotolidine test (as described in Standard Methods, 39). Buffers were considered chlorine demand-free and suitable for use when the orthotolidine test was negative.

iv. Chlorine determinations: The free residual chlorine content of all stock and treatment solutions was determined by amperometric titration (39). The chlorine residual of treatment and control solutions immediately before and after each treatment experiment was determined by the N,N, diethyl-p-phenyldiamine (DPD) colorimetric method (39).

v. Neutralizer solution. To neutralize the effect of chlorine a 10% solution of sodium thiosulfate ($Na_2S_2O_3$) in 0.001 M phosphate buffer (KH_2PO_4-Na_2HPO_4) pH 7.4 was prepared, filtered (0.22 nm membrane) and stored at 4°C. The solution was autoclaved the day of the experiments.

vi. Chlorine treatment conditions: The chlorine demand of the HAV solution was determined by both amperometric and DPD methods to be 0.1-0.2 mg/L free residual chlorine. Treatment solutions were prepared at pH 7.0 with the desired free residual chlorine (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) plus 0.2 mg/L to satisfy the demand of the HAV solution. Treatment conditions were 5°C for 15, 30 or 60 min with gentle agitation in a oscillating water bath.

vii. Chlorine treatment procedure. A scaled-down system similar to that of Scarpino et al. (19) was used to conserve the volume of infectious HAV needed. On the day of each experiment, sufficient chlorine was added to CDF buffer to give the desired chlorine concentration. Aliquots of the CDF buffer solution having the desired chlorine residual were placed in beakers labeled "Test," "Chlorine Control" and "Temperature Control," respectively. Another beaker was labeled "Virus Control" and filled with an equal volume of the CDF buffer without any added chlorine. The beakers were allowed to "soak" at room temperature for 1 hour to satisfy any chlorine demand associated with the beakers. After "soaking" the contents of each beaker were discarded, replaced with a similar volume of CDF buffer solution, placed in a shaking 5°C water bath and equilibrated for 30 min until the desired temperature was reached. At the beginning and end of each inactivation experiment, 10 mL was removed from the "Chlorine Control" beaker and the residual chlorine determined. Partially purified HAV was added to the "Virus Control" and "Test" beakers which were agitated constantly throughout the contact period. At the end of the contact period 5 mL was removed from the "Virus Control" and "Test" beakers, diluted (1:2) with demand-free water and the residual chlorine determined. The reaction was stopped by addition of 0.1 mL solution of sodium thiosulfate neutralizing solution immediately after removal of the 5 mL for the chlorine determination. The remaining reaction mixtures were evaluated in vivo as described below.

D. Radioimmunoassay (RIA). Treated (HOCl) and untreated preparations of HAV were examined by the micro solid phase radioimmunoassay system described by Purcell and coworkers (40). Immunoglobulins (IgG) were isolated from plasma or serum by precipitation with sodium sulfate (18% final concentration) and the precipitate resuspended in saline (original

volume). This IgG preparation served as a source for coating and ^{125}I-labeled antibody reagents. This method involves the coupling of the unlabeled immunoglobulin containing anti-HAV to the insoluble polyvinyl matrix of a microtiter "U" well. These coated wells can then extract immunologically reactive HAV Ag from the treated (HOCl) and control HAV preparations. Detection of the bound antigen is measured following interaction with a radiolabeled ^{125}I-IgG preparation containing anti-HAV. The amount of reactive HAV Ag can be quantitated by end-point dilution. Antibodies to HAV were detected by RIA using the HAVAB test kits (Abbott Laboratories, Inc., North Chicago) following the manufacturer's procedure.

E. In vivo assay of infectivity.

Preliminary evaluation of partially purified HAV preparation(s) for infectivity was undertaken before the effect of chlorine on HAV was examined. For each treatment condition selected, marmosets entered baseline studies as described in A.i. above. The treatment groups consisted of 4 to 6 marmosets inoculated intramuscularly (IM) with 1 mL of chlorine-treated HAV suspension and the control groups consisted of 2 to 5 marmosets inoculated IM with an equivalent volume of untreated HAV suspension. Animals receiving chlorine treated inoculum were monitored for an additional 30 days (total of 150 day experimental period), since partial inactivation of HAV could result in extended incubation periods and/or inapparent disease. Animals were maintained and monitored as described in B.i.

SECTION 4

RESULTS

A. Preparation of HAV source material:

i. Infectious HAV pool: Portions of a fecal pool obtained from Ch 122 on 13, 14 and 15 dpi were thawed and processed as described in Materials and Methods (B. i.). Six marmosets were inoculated (intramuscularly with 0.5 mL or 1 mL) with this fecal pool. The inoculum induced hepatitis in 5/6 animals with a mean incubation period of 34 days and 6/6 animals developed antibodies to HAV (anti-HAV). This fecal pool served as the source of virus for all *in vivo* infectivity studies.

B. Preparation of partially purified HAV: Four different methods of preparing partially purified HAV from Ch 122 fecal pool C-1 material were evaluated.

i. Linear sucrose gradient method: 20 mL of Ch 122 feces was clarified (10,000 rpm/5°C/60 min/SW 27 rotor), the virus pelleted (38,000 rpm/5°C/2 hr/SW 41 rotor), pellet resuspended in 3 mL and 1 mL of this suspension layered on a 5% to 30% linear sucrose gradient (35,000 rpm/5°C/SW 41 rotor). High levels of antigenicity were detected in two fractions, the peak fractions (f4 and 5) were pooled, dialyzed (PBS, pH 7.8), ampulized and placed in liquid nitrogen storage.

ii. Cesium chloride - sucrose gradient method: 21 mL of Ch 122 fecal pool was clarified (13,000 rpm/5°C/30 min/JA 20 rotor), the virus pelleted (38,000 rpm/5°C/2 hr/SW 41 rotor), pellet resuspended in 3.1 mL and 1 mL of this suspension was layered on a 1.0 to 1.6 g/cc CsCl gradient (35,000 rpm/5°C/18 hr/SW 41 rotor). The peak of RIA activity was in f9 and 10 at a density of 1.325 g/cc as expected for HAV. The peak fractions (f9 and 10) were pooled, dialyzed, placed in ampules and stored. One mL of this preparation was then placed on 5% to 30% linear sucrose gradients (38,000 rpm/5°C/2 hr/SW 41 rotor) and peak activity was found in fractions 2 and 3. The fractions were pooled, dialyzed, ampulized and placed in liquid nitrogen storage.

iii. Freon-sucrose method: 30 mL of Ch 122 feces was extracted twice with Freon 113, the aqueous phase harvested (3,500 rpm/5°C/30 min/JA 20 rotor), filtered (pre filter/800 nm/450 nm) and the volume reduced to 6 mL by hydroextraction. Two mL of this preparation was placed on 5% to 30% linear sucrose gradients (26,000 rpm/5°C/3 hr/SW 27 rotor), peak activity was found in fractions 8, 9, 10, these fractions were pooled, dialyzed, ampulized and placed in storage.

iv. PEG-sucrose method: 30 mL of Ch 122 feces was clarified (8,000 rpm/5°C/30 min/JA 20 rotor), the supernatant made to 7% PEG 6000, the solution gently mixed periodically, then incubated at 4°C overnight. The precipitate was harvested (8,000 rpm/5°C/30 min/JA 20 rotor) and resuspended in 6 mL. 5% to 30% linear sucrose gradients were layered with 2 mL of the suspension, centrifuged (25,000 rpm/5°C/3 hr/SW 27 rotor) and peak activity was found in fractions 10 and 11. Peak fractions (f10 and 11) were pooled, dialyzed, ampulized and stored.

The linear sucrose gradient preparation (method i. above) resulted in a very poor HAV Ag yield and was considered unsuitable for these experiments. Table 1 presents a brief comparison of the HAV preparations made by the last three methods. However, the RIA runs are not exactly comparable since different labeled antibody sources were used. Since the Freon-sucrose or PEG-sucrose methods (preparations #1) show the most promise a second preparation of each was made. The second Freon preparation at a concentration value of 2.5 x (equals a 2.5 fold concentration, volume/volume; e.g., 30 mL of 20% feces thawed and final preparation volume of 12 mL) did not appear as effective as the second PEG-sucrose method (final preparation P/N ratios 7 vs 39, respectively). PEG-sucrose preparation #2 was made from 150 mL of 20% Ch 122 feces (as described above) ampulized, stored in liquid nitrogen.

TABLE 1. SUMMARY FOR HAV PARTIALLY PURIFIED PREPARATIONS[a]

Method	Assay Point	RIA Positive/Negative Ratio at Undil.	1:2	1:4	1:8
CsCl-Sucrose	Original 20% feces	37	28	nt[c]	nt
	CsCl peak fraction	35	nt	nt	nt
	Final prep. (10x)[b]	29	26	19	13
Freon Extraction #1	Original 20% feces	34	26	nt	nt
	Freon extract	35	nt	nt	nt
	Final prep. (10x)	45	36	25	17
Freon Extraction #2	Original 20% feces	36	29	28	nt
	Freon extract	18	12	nt	nt
	Final prep. (2.5x)	7	4	lt 2	lt 2
PEG Precipitation #1	Original 20% feces	39	30	22	15
	PEG precipitate	45	37	33	26
	Supernatant	2	6	5	3
	Final prep. 2.5x)	30	21	14	8
PEG Precipitation #2	Original 20% feces	38	32	26	18
	PEG precipitate	41	35	29	23
	Supernatant	lt 2	4	lt 2	3
	Final prep. (3x)	39	32	27	19

[a]for details see text
[b]10x equals a 10 fold concentrate (volume/volume); e.g. 30 mL 20% feces thawed and final preparation volume 3 mL
[c]nt - not tested
lt - less than

C. Infectivity of Partially Purified HAV: Results of the evaluation of PEG-sucrose preparation of HAV for infectivity in marmosets are shown in Table 2. A 1:5 dilution of the HAV preparation induced hepatitis in 3 of 4 animals, 1:10 dilution induced hepatitis in 2 of 4 and the 1:20 dilution in 2 of 3 animals with a mean incubation period of 30 dpi for the 7 marmosets which developed hepatitis. All eleven animals developed antibodies to HAV.

TABLE 2. INFECTIVITY OF HAV PREPARED BY PEG-SUCROSE TECHNIQUE

Inoculum Dilution[a]	No. Hepatitis / No. Inoculated	No. Seroconverted / No. Inoculated	Incubation Period[b]
1:5	3/4	4/4	28, 28, 28
1:10	2/4	4/4	28, 42
1:20	2/3	3/3	28, 28
TOTAL	7/11	11/11	

[a]Dilutions made with chlorine free PBS and 1.0 mL inoculated IM per marmoset.
[b]Days post inoculation, mean incubation period 30 days

The infectivity of the preparation, as reflected by 100% seroconversion (11 of 11) and the occurance of hepatitis in 64% (7 of 11) with a mean incubation period of 30 dpi indicated that the titer of the preparation was sufficient for the chlorine treatment experiments.

The infectivity titer of the PEG-sucrose preparation used in the disinfection experiments was performed in parts. In each chlorine treatment experiment the untreated control was a ten fold higher dilution of the preparation. The results of the titration are shown in Table 3. The ID_{50} titer of the 1:50 dilution used in the disinfection experiments was calculated (Reed & Meunch, 1938) to be $\geq 10^{-3.38}$ or approximately 1500 ID_{50}/mL.

TABLE 3. TITRATION OF PARTIALLY PURIFIED HAV IN MARMOSET MONKEYS[a]

Inoculum Dilution[b]	No. Hepatitis / No. Inoculated	No. Seroconverted / No. Inoculated
1:50	5/5	5/5
1:500	3/3	3/3
1:5,000	3/4	4/4
1:50,000	3/5	4/5
1:500,000	2/4	2/4

[a] Partially purified PEG-Sucrose preparation #2.
[b] Dilutions were made in chlorine demand free buffer and processed as controls with treatment groups for 30 min at 5°C with gentle agitation.

The chlorine demand of the PEG-sucrose partially purified preparation dilution of 1:50 selected for the chlorine treatments was 0.13 mg/L free residual chlorine (average of 5 determinations, range 0.09 to 0.16 mg/L) by amperometric method and 0.21 mg/L free residual chlorine (average of 10 determinations, range 0.10 to 0.33 mg/L) by DPD method. Therefore chlorine treatment solutions were prepared with 0.2 mg/L free residual chlorine more than the desired treatment levels to insure that the free residual desired was present after the chlorine demand of the virus preparation had been satisfied.

D. In vivo evaluation of chlorine treated HAV: The results of 11 treatment experiments and 2 control experiments are shown in Table 4. Partially purified HAV was diluted 1:50 in chlorine demand free buffer and treated for various periods (15, 30 or 60 min) with 0.5, 1.0, 1.5, 2.0 or 2.5 mg/L free residual chlorine at 5°C, pH 7.0 with gentle agitation. After the exposure period the chlorine was neutralized with sodium thiosulfate and the treated preparations were inoculated into marmosets. Untreated control HAV preparations diluted 1:50, 1:500, 1:5000, 1:50000 and 1:500000 were processed in a similar manner and inoculated into marmosets. The results of the control dilutions have been described above (Table 3). Treatment of HAV with 0.5 mg/L free residual chlorine in 3 experiments (15, 30 and 30 min exposure) resulted in

Table 4: INFECTIVITY OF CHLORINE TREATED PARTIALLY PURIFIED HEPATITIS A VIRUS IN MARMOSET MONKEYS (SAGUINUS SP.)[a]

Treatment mg HOCl/L	min.	No. Hepatitis / No. Inoculated	$\bar{x}$ inc.[b] period	No. Seroconverted / No. Inoculated	$\bar{x}$ Sero.[c] day
none	30	3/3	37	3/3	28
	30	2/2	45	2/2	48
		5/5 (100)[d]	40	5/5 (100)	36
0.5	15	1/5	71	2/5	85
	30	1/5	71	2/5	85
	60	0/4	--	0/4	--
		2/14 (14)	71	4/14 (29)	85
1.0	30	0/4	--	2/4	107
	30	1/4	--	1/4	48
	30	0/4	--	1/4	75
		1/12 (8)	--	4/12 (33)	77
1.5	30	1/5	70	1/5	70
	30	0/5	--	0/5	--
		1/10 (10)	70	1/10 (10)	70
2.0	30	0/4	--	0/4	--
	30	0/4	--	0/4	--
		0/8 (0)	--	0/8 (0)	--
2.5	30	0/5 (0)	--	0/5 (0)	--

[a]HAV virus was diluted 1:50 with treatment buffer, pH 7.0, containing the desired level of free residual chlorine (HOCl) at 5°C and treated for indicated time with gentle agitation.
[b]Mean incubaton in days.
[c]Mean seroconversion in days.
[d]( ) Percentage which developed hepatitis or seroconverted.

14% (2/14) of the animals developing hepatitis (vs 100% of the controls) and 29% (4/14) of the animals developing antibodies to HAV (vs 100% of the controls). In addition the incubation period (71 days) and seroconversion time (85 days) were longer than those of the controls (40 and 36 days respectively). HAV treated with 1 mg/L free residual chlorine in 3 experiments (30, 30 and 60 min exposure) induced hepatitis in 8% (1/12) of the animals and 33% (4/12) of the animals seroconverted. Treatment with 1.5 mg/L free residual chlorine in 2 experiments (30 min exposure) resulted in 10% (1/10) of the animals developing hepatitis and seroconverting. HAV treated with 2.0 mg HOCl (2 experiments with 30 min exposure) and 2.5 mg HOCl (1 experiment with 30 min exposure) prevented the development of apparent hepatitis and antibodies to HAV.

E. Immunogenic effect of chlorine treated HAV. Six animals which had been previously inoculated with chlorine treated HAV (4 with 2.0 mg and 2 with 2.5 mg/L free residual chlorine) were given two additional inoculations (at 14-day intervals) of HAV treated with 2.5 mg/L free residual chlorine (30 min.). None of these animals developed detectable anti-HAV antibody.

SECTION 5

DISCUSSION

Treatment of partially purified HAV with 0.5 to 1.5 mg/L free residual chlorine for 30 min decreased the incidence of overt hepatitis (10% to 14% vs 100% in controls) and seroconversion (10% to 33% vs 100% in controls). Because of limited numbers of animals and costs, it was impractical to titrate the HOCl treated HAV preparations, thus it is not possible to accurately determine the decrease in infectivity. The 1:50 dilution used for all treatment experiments contained on the order of $10^{3.38}$ MID_{50}/mL. In the 0.5 mg/L free residual chlorine treatment experiments only 14% (2 of 14) of the animals developed hepatitis and 29% (4 of 14) developed antibodies. Thus both the incidence of overt hepatitis and seroconversion are well below the 50% level, implying that the titer, after treatment, was less than 1 MID_{50}/mL or a minimal reduction in titer of $10^{3.38}$ MID_{50}/mL. The extended incubation and seroconversion periods observed in the treated groups are also indicative of low infective doses and have been seen repeatedly in titration experiments in marmosets. The protracted seroconversion periods (70 to 107 days) appear to be a true reflection of infection and multiplication of HAV with subsequent development of anti-HAV since immunization of animals 3 times with HAV, treated with 2.0 and 2.5 mg HOCl failed to induce any antibodies recognized as anti-HAV.

The development of overt hepatitis in 1 of 10 animals which received virus treated with 1.5 mg HOCl/mL for 30 min was probably due to the presence of small aggregates or clumps of HAV which were not completely penetrated by the HOCl. The fact that only this animal seroconverted suggests that the vast majority of single or small aggregates of virus were inactivated by 1.5 mg/L free residual chlorine.

While 8% (1/12) of the animals in the 1 mg/L free residual chlorine treatment group developed hepatitis, 33% (4 of 12) seroconverted. This seroconversion level is comparable to that of the 0.5 mg HOCl treatment group (29%; 4 of 14). If we exclude the 1 animal which developed hepatitis and seroconverted in the 1.5 mg HOCl treatment group, we see that 1.5, 2.0 and 2.5 mg/L free residual chlorine treatment essentially destroys the infectivity of HAV. Thus, the critical treatment level appears to be bewteen 1.0-1.5 mg/L free residual chlorine in these experiments. The levels of HOCl required to inactivate HAV within the environment, protected by fecal material, aggregated and dispersed in water are unknown.

These results, the decrease in incidence of disease and development of anti-HAV and the lengthening of the incubation period and appearance time of anti-HAV, indicate that treatment levels of 0.5 - 1.5 mg/L free residual chlorine for 30 min have inactivated most but not all HAV in the preparations. Concentrations of 2.0 and 2.5 mg/L free residual chlorine destroyed the infectivity.

It is interesting to note that an American Water Works Association Committee (41) recently recommended that appropriate disinfection of drinking water to ensure viral inactivation could be achieved by maintaining a free chlorine residual of 1.0 mg/L free residual chlorine for at least 30 min at water pH's of less than 8.0 Data from a number of investigations reviewed by the National Academy of Sciences (42) indicate that under these conditions enterovirus numbers were consistently reduced by two orders of magnitude in less than 5 min. The results of this investigation suggests that HAV may be considerably more resistant to chlorine than other enteroviruses.

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